No. PCT/US92/07381, filed August 28, 1992, and a continuation-in-part of United States
Patent Application Serial No. 08/182,117, filed January 27, 1994, now abandoned,which
is a continuation-in-part of International Application No. PCT/US92/06283, filed July 30,
1992, United States Patent Application Serial No. 07/750,579, filed August 28, 1991, now
abandoned, and United States Patent Application Serial No. 07/738,040, filed July 30,
1991, now abandoned, which is a continuation-in-part of United States Patent Application
Serial No. 07/559,955, filed July 31, 1990, now abandoned,which is a continuation-in-
part of United States Patent Application Serial No. 07/472,070, filed January 30, 1990,
now abandoned, which is a continuation-in-part of United States Patent Application Serial
No. 07/388,044, filed July 31, 1989, now abandoned."

10

IN THE CLAIMS:

Please add the following new claims:

38. (New)   The recombinant DNA sequence of claim 11, wherein the human thyroid
peroxidase is immunogenic to human thyroid peroxidase autoantibodies.

39. (New)   A vector which comprises the DNA sequence of claim 38.

20

40. (New)   A host cell transformed with the vector of claim 38.

REMARKS

Claims 11 to 15 are pending but rejected. Claim 37 has been withdrawn from
consideration. Claims 38 to 40 are newly added.

Claims 38 to 40 are supported by Example XI (Specification, pp. 48 - 60). The
specification, at p. 58, lines 6 - 8 states: "As a consequence of the mutation, a 'truncated'
human TPO protein is expressed which is secreted by the host cell rather than bound to its
membrane". A specific example of the vector of claim 39 is disclosed in the specification,
p. 58, lines 16 - 21. Specific examples of the host cells of claim 40 are CHO-TPO-MI-K
cells. The first full paragraph of p. 59 of the specification discloses the testing of the
secreted human thyroid peroxidase (hereinafter referred to as "hTPO") for its ability to

30

immunoprecipitate human anti-hTPO antibodies in. Hashimoto's serum and the result was illustrated in Figure 14B. As explained in the "Brief Description of the Drawings", Figure 14B shows the "Immunoprecipitation of mutated hTPO from clones of CHO-TPO-MI-K cells generated by limiting dilution. Immunoprecipitations were performed with serum from a patient with Hashimoto's thyroiditis with high anti-hTPO antibody levels. The specificity of the immunoprecipitation is shown by the inability of serum from a normal individual (CON) to precipitate the 105-101 kD doublet." (Specification, p. 14, first full para.).

The following addresses the items raised in the Office Action

10

Item 5

The specification on page 1 is hereby amended to reflect that all the parent U.S. patent applications are now abandoned.

Item 6

The undersigned thanks the Examiner for holding in abeyance the objections found in Sections 5, 6, 7, and 8 of Paper No. 12, pending notification of allowable subject matter.

20   Item 7

A copy of the unmarked "Combined Declaration for Patent Application and Power of Attorney" (hereinafter referred to as "Combined Declaration") executed by the inventor on December 14, 1995 is attached hereby as Attachment 1. This Combined Declaration identifies this application by its serial number and filing date. A copy of the Combined Declaration was filed on February 12, 1999 along with the Transmittal Form for a prior CPA to this patent application.

Please note that the prior Office Actions, such as Paper 12, has objected to the Combined Declaration for lacking in post office address. The inventor has moved and his new post office and residential addresses are the same. A new executed Combined
30   Declaration with the new address will be obtained.

Meantime, to reflect the inventor's current residential and post office address, submitted herewith is also a "Notification of Inventor's Current Residential and Post Office Address".

3

Please also note that prior Office Actions, such as Paper 12, has objected to the Combined Declaration for not claiming priority, under 35 USC 120, for PCT/US92/07381 and PCT/US92/06283. The undersigned just observed that PCT/US92/07381 was filed internationally on August 28, 1992, and claims a priority date of August 28, 1991. However, U.S. application serial number 08/196,082, which claims to be the PCT application's continuation, was filed on March 3, 1994. March 3, 1994 was more than 30 months, i.e., beyond February 28, 1994, after the priority date of the PCT application if a PCT Chapter II National Phase was pursued. The undersigned will investigate the matter, which will likely entail ordering the filewrapper of USSN 08/196,082 to see if there is an explanation for the late filing date which may still ensure priority claim. Due to this concern, the undersigned will also order the filewrapper of USSN 08/182,117 which claims priority of PCT/US92/06283. Thus, applicant respectfully requests that the Examiner holds in abeyance the request for a corrected Combined Declaration (reflecting PCT priority claims, current inventor's residential and post office address), and certified copies of both the above PCT applications, until applicant has been able to ascertain the priority claims.

Item 9

Claims 11 to 15 are rejected under 35 USC 103. In summary, the Examiner's position is that "...regardless of nucleic acids [in that they are NOT nucleic acids for encoding hTPO protein] and organisms involved, the references clearly teach the production of secreted proteins by transfecting host cells with cDNA encoding truncated protein as well as the advantages of secreted proteins thus one of ordinary skill in the art would have expected to be able to successfully produce secretable truncated proteins in view of the cited references." (Office Action, page 5, lines 2 - 6. Words within brackets are those of applicant's. Emphasis ours.) The Examiner deems that since DNA sequence to hTPO was known and the transmembrane domain of porcine thyroid peroxidase was known, it was within the purview of one of ordinary skill in the art to determine the transmembrane domain of the known hTPO. (Office Action, page 5, lines 14 - 18.

Applicant respectfully traverses the rejection. The references do not specifically teach nor suggest that a recombinant DNA encoding a secretable human thyroid peroxidase can be successfully produced. The Examiner is relying on the teaching regarding OTHER transmembrane proteins that are NOT hTPO to show that "one of ordinary skill in the art

would have expected to be able to successfully produce secretable truncated" hTPO protein (see the above quote of the Examiner). In other words, the Examiner uses the references to support the generalization that one skilled in the art would have expected that ANY transmembrane protein, when removed of their transmembrane region, would be secretable. Applicant respectfully submits that this is not the teaching nor suggestion of the prior art. Further, claims 16 to 18 require that the secreted hTPO be immunogenic to human autoantibodies to hTPO. Again, applicants respectfully submits that the references, alone or in combination, does not teach nor suggest that a truncated hTPO will be secretable, and least of all immunogenic to human autoantibodies to hTPO.

10   Applicant respectfully submits that the Examiner's position that one skilled in the art would have expected that ANY transmembrane protein, when removed of their transmembrane region, would be secretable (and immunogenic in the case of claims 38 to 40) is incorrect and cannot be used to reject the pending claims.

In support of applicant's position, submitted herewith is the following reference: B. Rapoport et al., "Critical Relationship between Autoantibody Recognition and Thyrotropin Receptor Maturation as Reflected in the Acquisition of Complex Carbohydrate" , *J. Clin. Endo. & Metabolism*, 81(7): 2525 - 2533 (1996), which is hereby attached as Attachment 2. This reference is hereinafter referred to as "Ref1".

Firstly, it should be noted that scientific papers or references disclosing the authors'
20   failure to achieve their goals (such as failure to produce secretable and/or immunogenic transmembrane proteins) are extremely rare, and likely nonexistent for patents/applications. People are unlikely to waste time and money to publicize or file patent applications on failures. Therefore, it is not surprising that the Examiner only found references that show success by the authors or inventors.

Ref1 is an article that shows and confirms the failure to produce a secretable ectodomain of a transmembrane protein and this protein's failure to recognize human autoantibodies to the full length protein. Ref1 reviews the progress for producing a secretable transmembrane human thyrotropin receptor (hereinafter referred to as "TSHR"). Firstly, Ref1 shows that even if the cDNA of a human transmembrane protein has been
30   cloned, sequenced and expressed, one skilled in the art would not have expected to be successful in generating a secretable recombinant form of the protein, least of all one that is immunogenic to autoantibodies. Ref1 shows that many skilled in the art have actually failed, despite numerous much motivated efforts, for 6 years to produce such a secretable

5

protein and Ref1 confirms that such a secretable protein cannot be produced, least of all a secretable protein that is immunogenic to patient's autoantibodies.

"An essential goal, if major progress is to be made in understanding the pathogenesis of Grave's disease at a molecular level, is the generation of large quantities of TSHR protein in a form recognized by the patients' autoantibodies. Because of the extremely low level of TSHR expression in thyroid tissue as well as the difficulty in obtaining large quantities of human tissue, the only hope is the generation of recombinant material.

10 Unfortunately, for the 6 yr since the molecular cloning of cDNA for human TSHR (17, 18, 29), the task has proven to be exceptionally difficult. As mentioned above (see introduction), a large effort by numerous laboratories using different expression system has tried to no avail. Many other attempts have been unreported. For example, in the past 5 yr we have tried four different epitope tagging approaches, only the last of which (6 H) is reported herein." (Ref1, p. 2531, under "Discussion" section, 1st para.).

It will be noted that reference 17 (cited in the above quote) was published in 1989 and discloses that TSHR has been cloned, sequenced, and expressed. Ref 17 is Nagayama

20 Y., et al., "Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor", *Biochem Biophys Res Commun.*, 165: 1184-1190 (1989). *porcine* This publication is two years after the publication in 1987, of Magnusson et al which is cited against the pending application. Yet, the numerous very much motivated scientific efforts from 1989 to 1996 (which overlaps the priority claims of the pending application of 1989 to 1994) has proven unavailing in producing a recombinant TSHR that is recognized by autoantibodies. The reason for this failure is TSHR without its transmembrane domain (TSHR-ectodomain or "TSHR-ECD" for short) is not secretable and possesses immature carbohydrate that is not recognized by autoantibodies in patients' sera. This is contrary to the Examiner's position that once a transmembrane protein's cDNA has been cloned, one

30 skilled in the art can expect to produce its secretable form by removing its transmembrane region.

Ref1 confirms that: "The present data describe our experience over the past 5 yr in this endeavor. Our findings support evidence from another laboratory that the TSHR-ECD

6

is not secreted by transfected mammalian cells (7), but is retained as a protein in the soluble fraction of the cell." Ref1, p. 2525, right col., second full para., first two sentences. The cited reference (7) is: Harftst E., et al., "Characterization of the extracellular region of the human thyrotropin receptor expressed as a recombinant protein", *J. Mol. Endocrinol.* 9: 227 - 236 (1992).

Ref1 presents the many factors that may cause a transmembrane protein to be non-secretable when it is truncated (Ref1, paragraph bridging pp 2531 - 2532) . Ref1 also indicates that the causes may be unclear. The Examiner has sidestepped these obvious factors. A factor which would have been obvious (because it is such common sense) to

10   one skilled in the art even at the time of filing of the application is "... if a protein, perhaps because of hydrophobic or charge interactions [due to its truncation], is unable to enter the Golgi complex." Ref1, p. 2532, left col., lines 7 - 10.
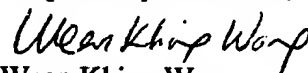
Even at the time of filing of the application, one skilled in the art would also have realized that the complex interactions of chemical groups or removal thereof from a protein due to its truncation may cause it not to be immunogenic to autoantibodies to the full length protein.

Ref1 also discloses that the truncation prevents the recombinant TSHR protein's acquisition of complex carbohydrate which is critical for autoantibody recognition: "Thus, even after considerable purification by lectin affinity chromatography, receptor ECD with

20   immature high mannose carbohydrate was incapable of neutralizing TSHR autoantibodies in any of the [patients'] sera tested." (Ref1, p. 2532, second full para., 2nd and 3rd sentences).

In view of the above discussion, applicants respectfully submit that at the time of filing of the application, one of ordinary skill in the art would not have expected to be able to successfully produce secretable truncated hTPO, least of all one that is immunogenic to hTPO autoantibodies. Therefore, the claimed invention is nonobvious and applicant respecfully submits that the Examiner's rejections under 35 USC 103 have been overcome.

Respectfully submitted,

30

Wean Khing Wong
Wean Khing Wong
Attorney for Applicant
TEL: (323) 257-8822
TEL: (323) 259-0447
Registration No. 33,561

7

Ker 1

Critical Relationship between Autoantibody Recognition and Thyrotropin Receptor Maturation as Reflected in the Acquisition of Complex Carbohydrate*

BASIL RAPOPORT, SANDRA M. McLACHLAN, AYUMU KAKINUMA, AND GREGORIO D. CHAZENBALK

Thyroid Molecular Biology Unit, Veterans Administration Medical Center, and the University of California, San Francisco, California 94121

ABSTRACT

Generation of large amounts of recombinant TSH receptor (TSHR) protein capable of recognition by TSHR autoantibodies is a goal of clinical importance. We expressed in Chinese hamster ovary cells the human TSHR ectodomain (ECD) with a carboxyl-terminus six-histidine tag. After transgene amplification, expressing clones were selected by nickel chelate chromatography in combination with [^{35}S]methionine precursor labeling. An approximately 74-kDa protein was detected in the culture medium, and larger quantities of an approximately 68-kDa protein were found in the cell soluble fraction. Immunoblot analysis with a rabbit antiserum revealed that most of the TSHR-ECD was not secreted, but was retained within the soluble fraction of the cell. Nickel chelate chromatography was not effective in purifying significant quantities of this material. In contrast, with Concanavalin A, but not with wheat germ agglutinin, an approximately 50-fold purification of TSHR-ECD was achieved from the cell

soluble fraction. Surprisingly, this affinity-enriched TSHR, containing high mannose carbohydrate, was not recognized by human TSHR autoantibodies in sera from six individuals. By ion exchange chromatography, the autoantibody-neutralizing TSHR in the cell supernatant fraction was found to be nonidentical with TSHR-ECD protein recognized by antisera from immunized animals.

The present data indicate the critical relationship between autoantibody recognition and TSHR maturation as reflected in the acquisition of complex carbohydrate. Nonsecretion of the TSHR-ECD appears to be related to the specific protein rather than to the glycosylation apparatus of the host cell. Antibodies from immunized animals may be ineffective in monitoring purification of autoantigen-competent TSHR. Finally, the data explain why soluble recombinant TSHR generated in many expression systems is not recognized satisfactorily by human autoantibodies. (*J Clin Endocrinol Metab* **81**: 2525–2533, 1996)

$\mathbf{P}$URE, CONFORMATIONALLY intact antigens are the key to understanding the pathogenesis of Graves' disease and Hashimoto's thyroiditis (reviewed in Ref. 1). These two diseases, the most common organ-specific autoimmune diseases affecting humans, are both characterized by IgG class autoantibodies to thyroid-specific, cell surface antigens. Autoantibodies to thyroid peroxidase (TPO), expressed on the apical surface of the thyrocyte, are an invariable marker of active Hashimoto's thyroiditis and are also present in 70–80% of patients with active Graves' disease (reviewed in Refs. 2 and 3). Autoantibodies to the TSH receptor (TSHR), expressed on the basolateral surface of the thyrocyte (4), are directly responsible for the clinical manifestation of Graves' disease (reviewed in Ref. 5).

It has been frustrating that information on the molecular interaction between TSHR autoantibodies and TSHR antigen lags far behind that for TPO autoantibodies and TPO. Two primary factors are responsible for this disparity. First, unlike TPO autoantibodies in patients' sera, TSHR autoantibodies are present in minute concentrations (reviewed in Ref. 1; 6,7). Second, in comparison to TPO (reviewed in Refs. 2 and

3), it has been more difficult to produce large quantities of conformationally intact TSHR, either the holoreceptor or its ectodomain (ECD), in bacterial (4, 7–11), baculovirus (10, 12–15), adenovirus (16), and yeast (Rapoport, B., unpublished data) expression systems.

The difficulty in producing satisfactory TSHR antigen by the approaches described above contrasts with fact that the human TSH holoreceptor, stably expressed in mammalian cells, is clearly functional, of high affinity (17–19), and recognized by autoantibodies (20, 21). However, the low expression level and apparent instability of the receptor on these cells has made purification from this source impractical. Given all of these difficulties, a logical alternative approach is to attempt TSHR-ECD expression as a secreted protein from stably transfected mammalian cells.

The present data describe our experience over the past 5 yr in this endeavor. Our findings support evidence from another laboratory that the TSHR-ECD is not secreted by transfected mammalian cells (7), but is retained as a protein in the soluble fraction of the cell. We show that this fraction does contain TSHR that is capable of neutralizing human autoantibodies. However, this functional autoantigen is present in only trace amounts. Most of the receptor is present as a protein with immature, high mannose carbohydrate that is not recognized by autoantibodies. These data reveal that full maturation of the TSHR-ECD, as reflected by the acquisition of complex carbohydrate, is critical for autoantibody recognition.

Materials and Methods

Construction of plasmid for high level expression of the human TSHR-ECD

The construction of a plasmid containing the complementary DNA (cDNA) for the TSHR-ECD with an oligonucleotide cassette coding for a three-glycine spacer and six-histidine (6 H) tag at its carboxyl-terminus (amino acid 418) has been described previously (15). The 1.3-kilobase TSHR-ECD-6 H cDNA fragment, excised with *BglII* (blunted with Klenow polymerase) and *SalI*, was inserted into the *XbaI* (blunted) and *SalI* sites in pSV2-DHFR-ECE-TPO (22, 23) after removal of the *SalI-XbaI* insert. The new plasmid was termed pSV2-DHFR-ECE-TSHR-EC-6H.

Chinese hamster ovary (CHO) dhfr cells (CHO-DG44; kindly provided by Dr. Robert Schimke, Stanford University, Palo Alto, CA) were propagated in Ham's F-12 medium supplemented with 10% FCS, penicillin (100 U/mL), gentamicin (50 µg/mL), and amphotericin B (2.5 µg/mL). Cells were transfected with pSV2-DHFR-ECE-TSHR-EC-6H plasmid (20 µg) by the calcium phosphate precipitation method (24). Stably transfected cells were selected in thymidine-, guanine-, and hypoxanthine-free Ham's F-12 medium supplemented with 10% dialyzed FCS and antibiotics as described above. Methotrexate (MTX) was added to this selective cell culture medium at an initial concentration of 20 nmol/L, and surviving cells were expanded. The MTX concentration was sequentially increased, as described in *Results*, until a final concentration of 10,000 nmol/L (10 µmol/L) was reached.

Selection of clones expressing TSHR-ECD with nickel-nitrilo-tri-acetic acid (Ni-NTA) resin

CHO cells to be tested for TSHR-ECD expression were grown to confluence in 60-mm diameter dishes. After rinsing with phosphate-buffered saline (PBS), cells were preincubated (1.5 h, two times) in DMEM-H21 methionine-free medium containing 10% dialyzed FCS. The cells were then pulsed (4 h at 37 C) in fresh medium supplemented with about 150 µCi [^{35}S]methionine. After aspiration of the medium and rinsing the cells once in PBS, chase was performed overnight in standard F-12 medium with 10% FCS.

Ni-NTA resin (Qiagen, Chatsworth, CA; 50 µL of a 50% slurry) was added to 1.5 mL of the chase medium in an E tube and tumbled for 1 h at room temperature. The resin was washed three times with 1 mL buffer B (8 mol/L urea, 0.1 mol/L Na phosphate, and 0.01 mol/L Tris, pH 6.3) with the use of a microfuge. The protein was then released from the resin by incubation for 10 min at room temperature with 40 µL buffer B containing 0.1 mol/L ethylenediamine tetraacetate (EDTA). Aliquots (20 µL) in Laemmli buffer containing 0.7 mol/L β-mercaptoethanol (25) were subjected to SDS-PAGE. Mol wt markers (Bio-Rad Laboratories, Richmond, CA) were run in parallel with the samples. Gels were dried, and radiolabeled proteins were visualized by autoradiography on Kodak XAR-5 x-ray films (Eastman Kodak Co., Rochester, NY).

Expression of TSHR-ECD in CHO cells

Up to 75 confluent 10-cm diameter dishes of CHO cell clone 17P-9, chosen for maximum TSHR-ECD expression (see *Results*), were rinsed once with PBS and scraped into ice-cold lysis buffer (3 mL/dish of cells). Lysis buffer was 10 mmol/L Tris, pH 7.4, with phenylmethylsulfonylfluoride (100 µg/mL), aprotinin (1 µg/mL), leupeptin (1 µg/mL), and pepstatin A (2 µg/mL; all from Sigma Chemical Co., St. Louis, MO). The cells were homogenized on ice with a Polytron homogenizer (Brinkmann Instruments, Westbury, CT; three pulses of 10 s each) and centrifuged for 1 h (4 C; 100,000 × *g*), and the supernatant (soluble fraction) was saved.

When indicated, the cell soluble fraction (before or after lectin affinity purification) was chromatographed on a Mono-Q column (Pharmacia, Piscataway, NJ) equilibrated with 20 mmol/L Tris, pH 7.4. After rinsing, the column was developed with a linear gradient of the same buffer containing 0-1000 mmol/L NaCl (15 mL at 1 mL/min). Fractions were used for enzyme-linked immunosorbent assay (ELISA) or TSHR autoantibody neutralization studies (see below).

TSHR-ECD lectin affinity purification

Concanavalin A (Con A-Sepharose, Pharmacia, Piscataway, NJ) was prewashed in 20 mmol/L Tris (pH 7.4), 0.5 mol/L NaCl, 1 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, and 1 mmol/L MnCl$_2$ and added to the cell soluble fraction (see above; 1 mL of a 70% suspension/100 mL). After stirring for about 1.5 h, the resin was recovered by centrifugation (3 min, 100 × *g*), washed six times with the same buffer, and eluted in batch in 6-8 mL 20 mmol/L Tris, pH 7.4, supplemented with 0.4 mol/L α-D-methylmannoside and 5 mmol/L EDTA (30 min at room temperature). Affinity purification with agarose wheat germ lectin was performed similarly, except that 2 mL suspension were added per 100 mL soluble fraction, and the elution sugar was 0.5 mol/L N-acetylglucosamine. Eluted samples were dialyzed overnight in approximately 200 vol 20 mmol/L Tris, pH 7.4 (4 C).

Enzymatic deglycosylation of the TSHR-ECD protein

After lectin affinity chromatography, material released from Con A and wheat germ was dialyzed extensively against 20 mmol/L Tris (pH 7.5) and 5 mmol/L EDTA and then processed according to the protocol of the manufacturer (New England Biolabs, Beverly, MA). In brief, 27-µL aliquots of sample were denatured for 10 min at 100 C in 0.5% SDS-1% β-mercaptoethanol. N-Glycosidase F digestion (100 U for 2 h at 37 C) was performed in 50 mmol/L Na phosphate, pH 7.5, and 1% Nonidet P-40. Endoglycosidase H digestion (50 U for 2 h at 37 C) was performed in 50 mmol/L Na citrate, pH 5.5. Samples were then subjected to immunoblot analysis, as described above.

Immunoblots of TSHR-ECD

Samples were solubilized with Laemmli's sample buffer (25) with 5% β-mercaptoethanol and incubated at 37 C for 30 min. After electrophoresis on SDS-7.5% polyacrylamide gels, proteins were transferred (2.5 milliamps/cm^2, 1.0 h) with a Poly Blot Transfer system (American Bionetics, Hayward, CA) to ProBlott membranes (Applied Biosystems, Foster City, CA). The membranes were rinsed three times with 50 mmol/L Tris-HCl buffer, pH 7.5, and 150 mmol/L NaCl (TBS) and then rocked for 60 min with TBS containing 5.0% skim milk powder. Membranes were incubated overnight (4 C) with a rabbit polyclonal antibody against the TSHR-ECD generated in a baculovirus system (R8, kindly made available to us by Dr. J. P. Banga, Kings College School of Medicine, London, UK; 1:1000) in TBS. After rinsing, the membranes were incubated for 2 h at 37 C with alkaline phosphatase-conjugated goat antirabbit IgG (Fc fragment specific; Caltag, South San Francisco, CA; 1:400 dilution). The signal was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mmol/L Tris-HCl buffer, pH 9.5, containing 100 mmol/L NaCl and 5 mmol/L MgCl$_2$.

ELISA for TSHR-ECD

The approach was based on a TPO autoantibody ELISA previously described (26). In brief, duplicate aliquots of the soluble fraction (50 µL) of CHO cells expressing TSHR-ECD or untransfected CHO cells were used to coat ELISA plates (Immulon 4, Dynatech Laboratories, Chantilly, VA) by incubation with an equal volume of 0.05 mol/L bicarbonate buffer (pH 9.3). As an additional control, duplicate wells were coated with 5% BSA. ELISA wells were incubated (1 h at 37 C) with R8 rabbit antiserum to the TSHR-ECD (see above) diluted 1:1000 in Tris-buffered saline, pH 7.4, containing 1% BSA. Subsequently, ELISA plates were washed and incubated (30 min, 37 C) with goat antirabbit IgG conjugated to alkaline phosphatase (Cappel, Organon Teknika Corp., Westchester, PA) diluted 1:400 in 150 mmol/L NaCl containing 1% BSA. After washing and addition of substrate (*p*-nitrophenyl phosphate), optical densities (OD) were measured at 405 nm. Similar assays were performed using a mouse monoclonal antibody to the TSHR-ECD (12) (A7, also made available to us by Dr. Banga) diluted 1:1000 as described above, followed by goat antimouse IgG conjugated to horseradish peroxidase (Sigma; diluted 1:500). For these assays, color was developed using o-phenylene diamine and H$_2$O$_2$, and OD was measured at 492 nm. Background values for control wells coated with BSA, which were subtracted from test wells, were always less than 0.05 OD units.

Assay for TSHR-ECD neutralization of TSHR autoantibodies

A standard TSH binding inhibition (TBI) assay (21, 27) was modified to test for autoantibody neutralization. In brief, IgG was prepared from the sera of patients containing TSHR autoantibodies or from normal individuals by protein G affinity chromatography (Hi-Trap, Pharmacia). Samples to be tested were diluted 1:1 with IgG (at final concentrations up to 2 mg/mL) in PBS and incubated overnight at 4 C. The mixture was then added (1.5 h at 37 C) to monolayers of CHO cells expressing the wild-type TSHR on their surface (17) (0.23 mL/well in a 24-well cluster plate). After rinsing, the cells were incubated (2 h at 37 C) with [^{125}I]TSH in binding buffer, and specific binding was determined, as previously described (21, 27).

Results

Generation of clonal CHO cell line expressing the human TSHR-ECD

CHO-DG44 cells were stably transfected with the plasmid pSV2-DHFR-ECE-TSHR-EC-6H. Cells from the approximately 250 clones obtained were pooled and seeded at low density in selection medium. Twenty clones from individual cells, selected with cloning cylinders, were expanded and tested for TSHR-ECD expression by precursor labeling with [^{35}S]methionine and affinity purification from the culture medium of a protein of the expected size (~64–70 kDa). The negative control included in each screen consisted of similar cells expressing at high level (1 μg/mL) the ECD of TPO without a 6 H tag (22, 23). None of the clones was found to secret a specific protein on PAGE and autoradiography. Consequently, culture of all 20 clones was continued in the presence of 20 nmol/L MTX to initiate amplification of the TSHR-ECD transgene.

After a further 3 months in culture, with a progressive increase in the MTX concentration to 80 nmol/L, 3 of the 20 clones (no. 3, 10, and 17) were found to be positive relative to the negative control (Fig. 1A). Thus, specific bands were visible at about 74 kDa in medium and about 68 kDa in cells. The limiting dilution was repeated on the 3 clones, and the MTX concentration was increased 3- to 4-fold at 4- to 8-week intervals, the time taken to obtain rapidly growing, resistant cell lines. Specific protein production, in medium or in the cells, was monitored periodically over 13 months. Over the period of gene amplification, the increase in the specific protein secreted into the medium lagged behind the increase in protein observed within the cells. After 2 additional limiting dilutions, a clonal cell line (17P; Fig. 1B) was selected and cultured in increasing MTX concentrations up to 10 μmol/L (Fig. 1C). Another limiting dilution generated clone 17P-9, which was used for further studies.

Confirmation by bioassay of TSHR-ECD expression

In large scale experiments (e.g. using 50 dishes of 17P-9 cells or 500 mL conditioned medium), we could not purify by Ni chelate chromatography sufficient ECD protein to be distinguished from nonspecific proteins in stained gels. This was the case even when samples were treated with urea (8 mol/L final concentration). We, therefore, examined whether a functional TSHR-ECD was present in conditioned medium or in the cell soluble fraction. For this purpose we modified a standard TBI assay (21, 27) for TSHR autoanti-
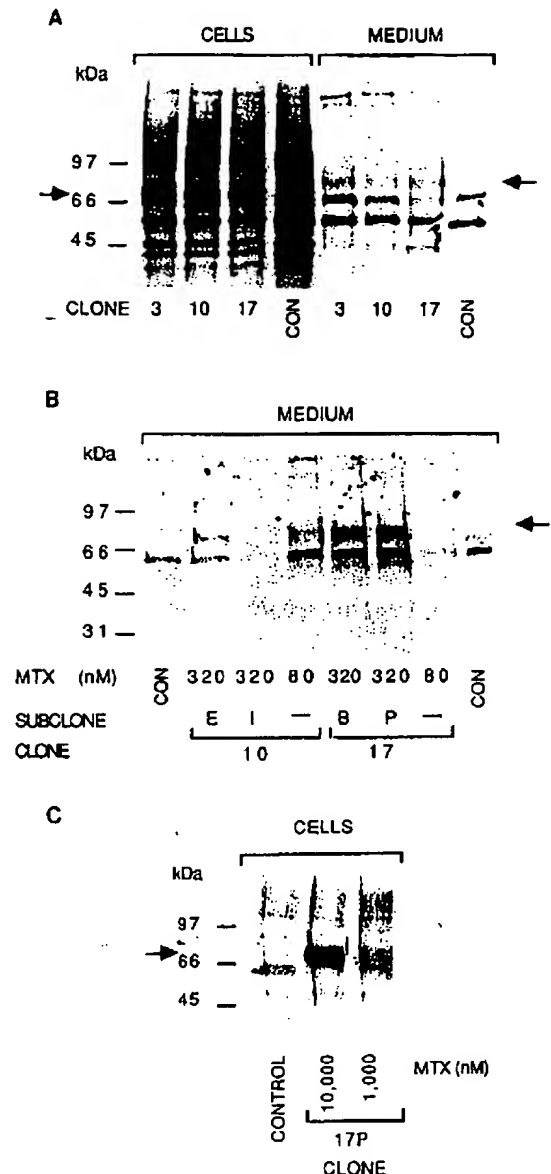

FIG. 1. Expression of specific protein as detected by precursor labeling of transfected CHO-DG44 cells. Cells in monolayer culture (35-mm diameter dishes) were pulsed for 4 h in 1 mL methionine-free medium containing 100–150 μCi [^{35}S]methionine (see Materials and Methods). As indicated, cells were either processed directly, or the medium was harvested after an overnight incubation in full medium (see Materials and Methods). Samples were subjected to Ni-NTA affinity purification (see Materials and Methods), and aliquots were electrophoresed on SDS-polyacrylamide gels under reducing conditions. A, Medium (pulse-chase) and cells (pulse) in clones stable at 80 nmol/L MTX. The arrows indicate the specific bands in the medium and cells relative to those in the TPO control (CON). Autoradiography of the dried 7.5% polyacrylamide gel was performed for 3 days. B, Representative clones after limiting dilution at 320 nmol/L MTX. Medium was processed after a pulse chase. The specific, approximately 74-kDa band in two clones is now seen to be equal in strength to that of the nonspecific 66-kDa band. Autoradiography (4–20% gel) was performed for 3 days. C, Specific, approximately 68-kDa band in pulsed cells (no chase) from clone 17P at 10 μmol/L MTX. Autoradiography (7.5% gel) was performed for 3 h.

RAPOPORT *ET AL.*

bodies. In this assay, IgG from patient or control serum is added to monolayers of CHO cells stably expressing the TSHR on their cell surface. Receptor occupancy by antibody inhibits subsequent [^{125}I]TSH binding to the cells, hence TSH binding inhibition. The modification introduced was preincubation of the IgG with conditioned medium or CHO cell soluble fractions before addition to the cells.

The 17P-9 soluble fraction partially and in some cases nearly completely (Fig. 2A) reversed the inhibitory effect of IgG with TSHR autoantibody activity on labeled TSH binding. In contrast, the soluble fraction from control CHO cells had no effect. Similar results were observed with IgG from six additional sera (TBI values of 15–69% were neutralized by a mean ± SD of 68%; range of neutralization, 32–100%). TBI activity in IgG preparations from four patients was not neutralized by the 17P-9 soluble fraction; three had very high TBI levels (96%, 81%, and 73%); the fourth TBI was 42%. TBI neutralizing activity in the 17P-9 soluble fraction could be diluted out with the soluble fraction from control CHO cells (Fig. 2B). Taken together, these data clearly established that TSHR-ECD functional activity was present as a soluble protein in the 17P-9 cells. No such activity was detected in the conditioned medium.

As a second approach to confirm TSHR-ECD expression by 17P-9 cells, we performed immunoblots on the soluble fraction and conditioned medium. For this purpose, we used a polyclonal rabbit antiserum to the TSHR-ECD. Although this antibody gave a very weak signal with the wild-type TSHR expressed in CHO cells (data not shown), it provided an exceptionally strong and specific signal for the TSHR in the soluble fraction of the 17P-9 cells selected for receptor overexpression (Fig. 3). Thus, a broad, approximately 68-kDa
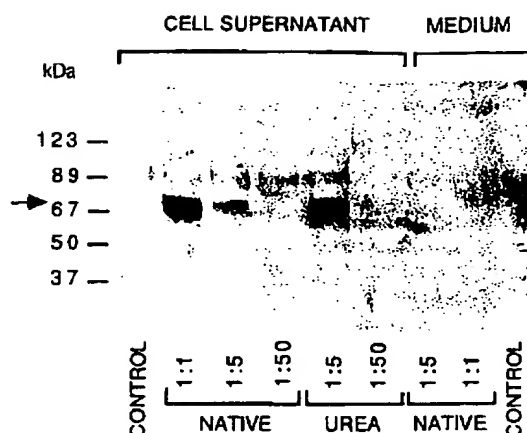

FIG. 3. Immunoblot of the TSHR-ECD in the 17P-9 cell soluble fraction and conditioned medium. The 100,000 × g soluble fraction (cell supernatant) or conditioned medium (see *Materials and Methods*) was subjected to SDS-PAGE (7.5%) under reducing conditions. After electrotransfer of proteins, membranes were exposed to a rabbit polyclonal antiserum to the human TSHR-ECD (see *Materials and Methods*). Where indicated, urea (final concentration, 8 mol/L) was added to the cell soluble fraction. Control, 100,000 × g supernatant fraction from CHO expressing the amplified TPO gene. The mol wt of markers run in an adjacent lane are shown.

band, the same size as that detected by Ni-NTA chromatography of [^{35}S]methionine-labeled products (Fig. 1), was detected only in 17P-9 cells and not in control CHO cells. Treatment of the soluble fraction with 8 mol/L urea moderately increased the specific signal (compare 1:5 dilutions; Fig. 3). TSHR-ECD was not detected in the conditioned culture medium using the rabbit antiserum. Even considering the 3.3-
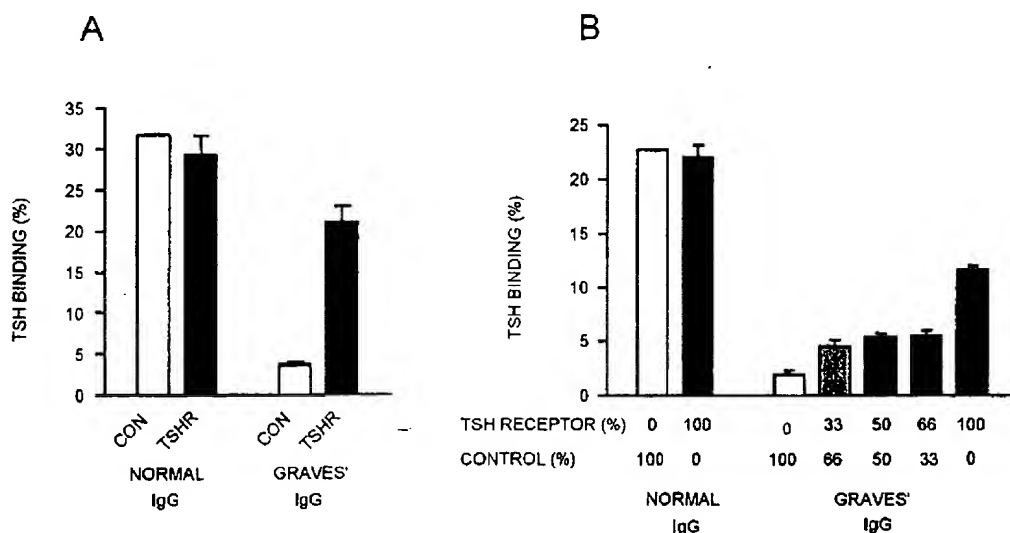
A

B

FIG. 2. The TSHR-ECD neutralizes the ability of TSHR autoantibodies to occupy the wild-type TSH holoreceptor. Purified IgG from patients or normal individuals (1.5 mg/mL final concentration) was preincubated overnight at 4 C with TSHR-ECD-containing 17P-9 cell supernatant fractions (*hatched bars*) or with supernatant fractions from control CHO cells (*clear bars*). The mixture was then added (1.5 h at 37 C) to monolayers of CHO cells expressing the wild-type TSHR on their surface. After rinsing, the cells were incubated (2 h at 37 C) with [^{125}I]TSH, and specific binding was determined (see *Materials and Methods*). The results shown are representative of six separate experiments using different TSHR extracellular domain preparations. A, IgG from a normal individual or an individual with TSHR autoantibodies mixed 1:1 with 17P-9 or control cell supernatant fractions. B, Separate experiment with IgG preincubated with different dilutions of the 17P-9 supernatant fraction. Dilutions were made in the supernatant fraction from control nonexpressing cells. Each *bar* indicates the range of determinations on duplicate dishes of cells.

fold greater dilution of the medium vs. cell soluble fraction (10 mL medium vs. 3 mL soluble fraction/dish of cells), it was evident that most of the TSHR-ECD was retained within the cells.

Lectin affinity chromatography of the TSHR-ECD

As described above, the Ni-NTA affinity matrix could be used to detect trace amounts of specific protein after precursor labeling, but it was ineffective for purifying relatively large amounts of native TSHR-ECD protein from 17P-9 cells. Because the TSHR is a glycoprotein, we performed lectin affinity chromatography on the 100,000 × g supernatant of the 17P-9 cells as well as on conditioned medium from cultures of these cells. As detected in immunoblots using the rabbit antiserum (Fig. 4), Con A removed much, in some experiments even all (Fig. 4A), of the TSHR-ECD in the 17P-9 soluble fraction. The receptor was recovered from the lectin with 0.4 mol/L α-methylmannoside. Relative to the protein recovered from Con A, the degree of TSHR purification was approximately 50-fold.

Affinity chromatography of the TSHR-ECD in the 17P-9 soluble fraction was much less efficient with wheat germ agglutinin than with Con A (Fig. 4B). Thus, there was a marked reversal between the two lectins in the ratio of receptor remaining in the cell soluble fraction and that recovered from the lectin. No detectable TSHR-ECD could be recovered with either lectin from the conditioned medium (data not shown). Enzymatic deglycosylation of the TSHR material recovered from both Con A and wheat germ agglutinin showed each to be sensitive to N-glycosidase F and endoglycosidase H (Fig. 4B). Sensitivity to the latter enzyme indicates that the carbohydrate on the soluble TSHR-ECD is of the high mannose variety.

Ion exchange chromatography of the Con A-enriched TSHR-ECD

The excellent enrichment of the TSHR-ECD on Con A encouraged us to attempt further purification of this material on a Mono-Q ion exchange column. A large number of protein peaks could be resolved (Fig. 5A). Immunoblot analysis

of the proteins in individual fractions revealed strong signals for the TSHR-ECD, as detected using a rabbit antiserum to the TSHR-ECD expressed in a baculovirus system. Surprisingly, in contrast to the excellent resolution of proteins on the Mono-Q column, TSHR immunoactivity was widely distributed throughout the fractions, with maximum activity eluting between 0.25–0.5 mol/L NaCl (Fig. 5B). This smeared distribution could not be attributed to TSHR degradation, because the material remained at 68 kDa in size. Overall, Mono-Q ion exchange chromatography yielded an additional approximately 20-fold purification in the fractions with peak TSHR immunoactivity. Together with the prior approximately 50-fold purification on Con A (see above), we attained an approximately 1000-fold purification of TSHR-ECD from the cytosolic fraction.

PAGE of proteins in the Mono-Q fractions revealed a wide diversity of proteins of different sizes in the different fractions (Fig. 5C). Disappointingly, despite the 1000-fold purification of the TSHR-ECD, none of the bands detected by direct staining were both 68 kDa in size and distributed in the column fractions in proportion to the immunoblot signals. That is, none of the visible bands was the TSHR-ECD. Based on the amount of protein applied to the gels, the level of TSHR-ECD protein was calculated to be no higher than 0.1–0.2 μg protein from 10^7 cells (10-cm culture dish). This very poor yield made any further purification of the material impractical.

Lack of autoantibody recognition of the Con A-enriched TSHR-ECD

As described above (Fig. 2), the 17P-9 cytosol contained immunologically active TSHR-ECD capable of neutralizing TBI activity. An important question arising from the Con A chromatography data was whether the TSHR recovered from the lectin represented this biologically active material. We, therefore, tested the ability of the lectin-enriched material to neutralize TSHR autoantibody activity in serum. The TSHR-ECD in the supernatant fraction was detected immunologically by ELISA using the rabbit antiserum (Fig. 6A) as well as by bioassay (neutralization of TBI activity; Fig. 6B). ELISA

FIG. 4. Lectin affinity chromatography of the TSHR-ECD. The 17P-9 100,000 × g soluble fraction (SUP; 100 mL) was stirred in batch with either Con A (A and B) or wheat germ agglutinin (WGA; B; see Materials and Methods). After extensive washing to remove unbound material, the Con A and the wheat germ agglutinin resins were treated for 30 min at room temperature with 0.4 mol/L α-D-methylmannoside or 0.5 mol/L N-acetylglucosamine, respectively. The eluted samples were dialyzed overnight in 20 mmol/L Tris, pH 7.4. Enzymatic deglycosylation of the TSHR-ECD protein (B) was performed with N-glycosidase F or endoglycosidase H digestion (see Materials and Methods). Aliquots were subjected to immunoblot analysis using the R8 rabbit antiserum (see Materials and Methods).
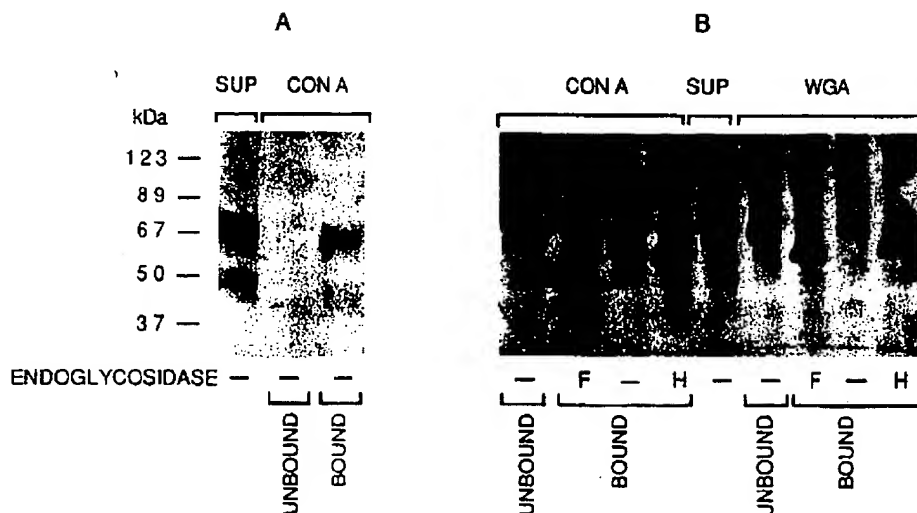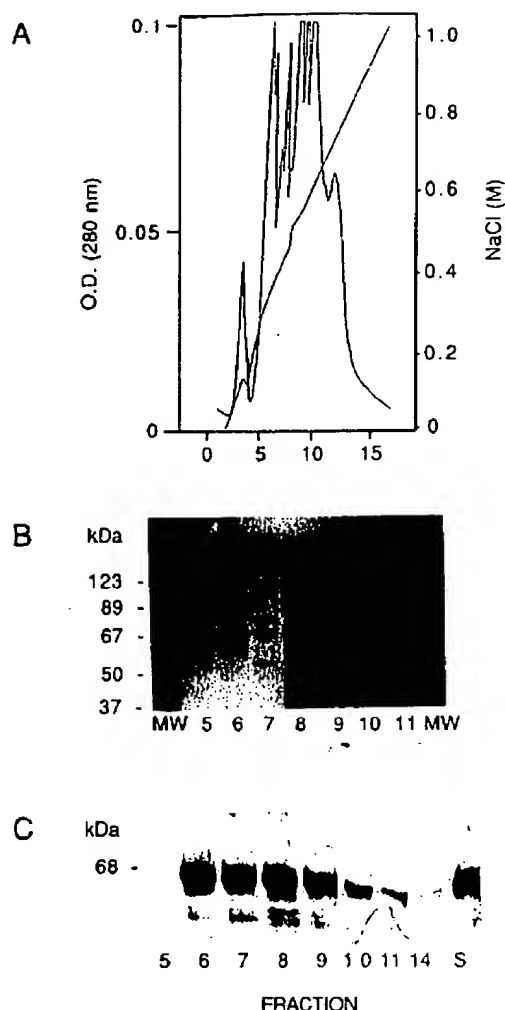
FIG. 5. Mono-Q ion exchange chromatography of the Con A-enriched TSHR-ECD. A, Proteins eluted with a 0-1 mol/L NaCl gradient (15 mL) were detected by absorption at 280 nmol/L. B, Coomassie blue stain of proteins in the Mono-Q column fractions. C, Immunoblot of TSHR-ECD in fractions eluted from the Mono-Q column. Aliquots of fractions were subjected to SDS-PAGE (7.5%) under reducing conditions. After electrotransfer of proteins, membranes were exposed to a rabbit polyclonal antiserum to the human TSHR-ECD and processed as described in *Materials and Methods*. The distortion in the gel in the later fractions is a consequence of the high NaCl concentration of the gradient. The mol wt of markers run in an adjacent lane are shown. S, soluble fraction before Mono-Q chromatography.

with the same rabbit antiserum using the Con A-enriched fraction revealed an increased concentration of TSHR-ECD (Fig. 6A), as expected from the immunoblot data (Figs. 4 and 5). Unexpectedly, the Con-enriched fraction did not neutralize TBI activity (Fig. 6B). A similar lack of TBI neutralization by the Con-enriched fraction was observed with all six sera tested. In these sera, TBI neutralization had previously been observed with the 17P-9 supernatant fraction. Thus, there was a dramatic dissociation between the rabbit antiserum and the human autoantibodies in their interaction with the TSHR-ECD. Recovery with the wheat germ agglutinin (Fig. 6A) was too low to permit interpretation of the activity of this material.
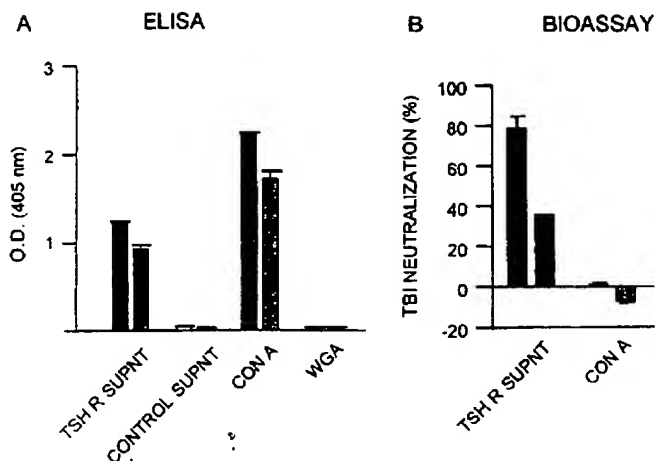

FIG. 6. Dissociation between human autoantibody and rabbit anti-serum recognition of Con A-enriched TSHR-ECD. A, The 100,000 × *g* supernatant fraction of 17P-9 (TSH R SUPNT) and untransfected cells (CONTROL SUPNT) as well as the Con A- and wheat germ agglutinin (WGA)-enriched material from the 17P-9 supernatant fraction were used to coat ELISA plates. Wells were screened with the rabbit antiserum to the TSHR-ECD (see *Materials and Methods*). B, The same material as that shown in A was preincubated with purified IgG from a patient with TSHR autoantibodies and with IgG from a normal individual without autoantibodies. TBI (see *Materials and Methods*) was calculated as: (TSH binding in normal IgG) − (TSH binding in patient's IgG) × 100. Maximum TBI activity in this experiment was 40%. Neutralization of the TBI value by preincubation with TSHR-containing material is expressed as: (TBI with control supernatant) − (TBI with 17P-9 material) × 100. For both the ELISA and bioassays, the *lighter bar to the right* in each pair of bars represents the same material diluted threefold. Each *bar* represents the mean and range of duplicate determinations.

Ion exchange chromatography of biologically active TSHR-ECD material

Because the TSHR-ECD partially purified by Con A was not the biologically active TSHR component in the 17P-9 cytosol that neutralized autoantibodies, we bypassed the Con A step and applied the proteins in the soluble fraction directly to the Mono-Q column (Fig. 7A). As for the lectin-enriched material, the TBI-neutralizing activity eluted in a broad smear (Fig. 7B), largely overlapping with the TSHR-ECD detected by ELISA using either the rabbit antiserum (Fig. 7C) or the murine monoclonal antibody A7 (Fig. 7D). This wide elution pattern contrasted with the sharp definition of the overall protein elution pattern (Fig. 7A).

Despite extensive overlap among Mono-Q fractions recognized by both human autoantibodies and sera from immunized animals, it is important to note that these two patterns were not identical. Thus, significant bioreactivity eluted initially at about 0.25 mol/L NaCl (see fraction 5 in the representative experiment shown (Fig. 7B), preceding recognition of TSHR material by the rabbit and mouse antisera (Fig. 7, C and D). Peak recognition of the TSHR-ECD by human autoantibodies was consistently observed one or two fractions earlier than with the rabbit or murine antibodies. It should be appreciated that because of the very low TSHR concentration as well as the inability of serum from immunized animals to detect the TSHR in fraction 5, TBI neutral-
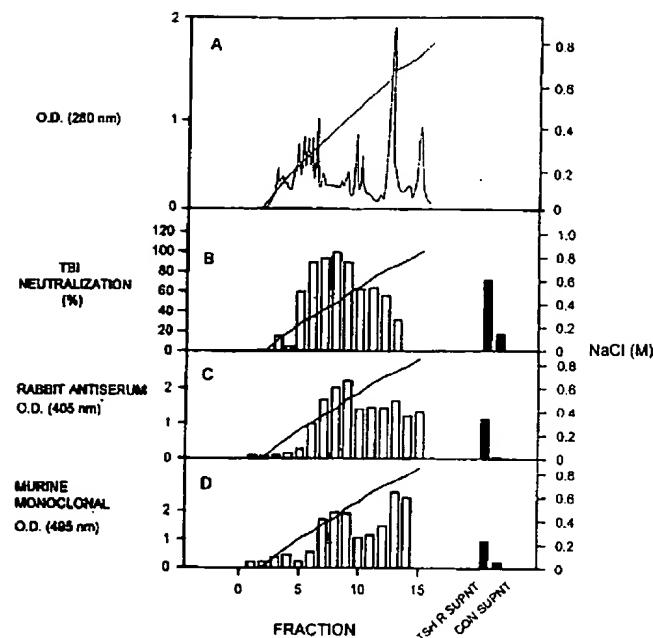
FIG. 7. Nonidentity of TSHR-ECD recognized by human autoanti-bodies and antibodies from immunized animals. The 100,000 × g supernatant fraction from 17P-9 cells was applied to a Mono-Q ion exchange column and eluted with a NaCl gradient. A, Proteins eluted with a 0–1 mol/L NaCl gradient (15 mL) were detected by absorption at 280 nmol/L. B, Neutralization by individual fractions of TBI ac-tivity (for calculation, see Fig. 6; maximum TBI activity in this ex-periment was 33%). C, ELISA of individual fractions with a rabbit antiserum (R8) to the TSHR-ECD generated in a baculovirus system. D, ELISA of individual fractions with a mouse monoclonal antibody (A7) to the TSHR-ECD generated in a prokaryotic system. TSH R SUPNT, 100,000 × g supernatant fraction of 17P-9 cells; CON SUPNT, same fraction of untransfected cells.

ization is the only approach able to detect the TSHR in this fraction.

A final observation from these studies was the major dif-ference in recognition patterns of TSHR-ECD with the rabbit antiserum and the murine monoclonal antibody. Thus, the rabbit antiserum detected TSHR-ECD in a single, very broad peak (Fig. 7C). In the same FPLC fractions, the murine mono-clonal antibody detected two peaks of TSHR-ECD (Fig. 7D). From data on Con A-enriched TSHR-ECD (Fig. 5C), it is likely that both rabbit and murine antibodies recognize vari-ants of the high mannose form of the protein.

Discussion

An essential goal, if major progress is to be made in un-derstanding the pathogenesis of Graves' disease at a molec-ular level, is the generation of large quantities of TSHR pro-tein in a form recognized by patients' autoantibodies. Because of the extremely low level of TSHR expression in thyroid tissue as well as the difficulty in obtaining large quantities of human tissue, the only hope is the generation of recombinant material. Unfortunately, in the 6 yr since the molecular cloning of cDNA for human TSHR (17, 28, 29), the task has proven to be exceptionally difficult. As mentioned above (see introduction), a large effort by numerous labo-

ratories using different expression systems has been tried to no avail. Many other attempts have been unreported. For example, in the past 5 yr we have tried four different epitope tagging approaches, only the last of which (6 H) is reported herein.

To overcome this frustrating roadblock, it is vital to un-derstand the reason(s) for this lack of success, rather than attempting further hit or miss approaches. The present study provides new and important insight into this process. It should be appreciated that the A subunit (~54 kDa) in the human TSHR-ECD contains about 20 kDa of carbohydrate (4); Chazenbalk, GD, McLachlan, SM, Nagayama, Y, and Rapoport, B., unpublished observations), nearly 40% of its mass! It is, therefore, not surprising that most investigators using prokaryotic expression systems have failed to generate TSHR of the correct conformation. This high carbohydrate content as well as possible heterogeneity in the carbohydrate moieties are likely reasons for the very broad elution pattern of the TSHR that we observed on ion exchange chromatog-raphy. Incidentally, the inability of autoantibodies in pa-tients' sera to recognize TSHR fragments expressed in bac-teria by cDNA fragment libraries has been interpreted as evidence for the conformational nature of the autoantibody epitopes (30). Clearly, this conclusion cannot be made from these data, for two reasons: 1) the use of nonglycosylated protein fragments, and 2) the exceptionally low levels of autoantibody in patients' sera (reviewed in Ref. 1; 6, 7). Stronger evidence for the conformational nature of the epitopes for TSHR autoantibodies is provided by data on chimeric TSH-LH/CG receptors (reviewed in Ref. 31).

The difficulty in producing a functional TSHR with bac-ulovirus vectors in eukaryotic insect cells (at least in our laboratory) is more of a disappointment. Lack of success for the TSH holoreceptor (13, 19) (Rapoport, B., unpublished data) has been attributed to its large, relatively hydrophobic, seven-membrane-spanning regions. However, expression of the more hydrophilic ECD has also been extremely prob-lematical. Vectors using the very late polyhedrin promoter generate relatively large amounts of ECD, but recognition by TSHR autoantibodies is poor (10, 12, 13). Use of a baculovirus vector with an earlier promoter to facilitate better glycosy-lation does produce an ECD recognized by autoantibodies, but only in trace amounts (15). Without the development of more effective vectors and host cells, the baculovirus system does not seem a viable option for a protein as complex and heavily glycosylated as the TSHR.

Mammalian eukaryotic cell expression systems, there-fore, seem to provide the best hope for generating rea-sonable quantities of functional TSHR antigen. There is no difficulty in expressing a functional, high affinity holore-ceptor on the surface of CHO cells in numbers much greater than those in thyroid tissue (reviewed in Ref. 31). As shown in the present studies, however, the TSHR-ECD expressed in the same cells is 1) largely nonsecreted and 2) contains predominantly high mannose carbohydrate that is well recognized by sera from TSHR-immunized animals, but not by human autoantibodies.

The reason for the inability of mammalian cells to secrete the TSHR-ECD (present report and Ref. 7) is unclear. Similar observations have been made for the closely related LH/CG

receptor (32, 33), although other investigators have reported the secretion of truncated LH/CG receptors (34, 35). Whether the high mannose carbohydrate noted in nonsecreted glycoprotein hormone receptor ECDs is primary or secondary to nonsecretion is unknown. Failure of an abnormal protein to fold correctly may lead to retention in the endoplasmic reticulum in a high mannose form (36). Alternatively, the same end point could occur if a protein, perhaps because of hydrophobic or charge interactions, is unable to enter the Golgi complex. We consider less likely the suggestion (14) that the expression of recombinant TSHR overwhelms the glycosylation capacity of the cell. CHO cells are clearly able to express large numbers of TSH holoreceptors with complex carbohydrate.

The inescapable conclusion, therefore, is that it is the protein, and not the expression system, that is producing the present difficulty. One approach taken to overcome this problem is to express in mammalian cells a fusion protein between the TSHR-ECD and part of another protein that may facilitate secretion. There is a preliminary report of success with the TSHR-ECD fused to portions of the CD4 and CD8 molecules (37). A similar approach by our laboratory, using the hinge, CH2, and CH3 regions of human IgG, has not led to promising results (Chazenbalk, G. D., S. M. McLachlan, and B. Rapoport,, unpublished data).

With respect to the TSHR as an autoantigen, the present data provide valuable new insight into recognition of the receptor by TSHR autoantibodies. As mentioned above, TSHR autoantibodies (reviewed in Ref. 31) as well as TSH (38) recognize highly conformational, discontinuous epitopes. It is now evident that full maturation of the TSHR-ECD, as reflected by the acquisition of complex carbohydrate, is also critical for autoantibody recognition. Thus, even after considerable purification by lectin affinity chromatography, receptor ECD with immature high mannose carbohydrate was incapable of neutralizing TSHR autoantibodies in any of the sera tested. In contrast, the unpurified 17P-9 cytosolic fraction contained sufficient antigen to neutralize TBI activity in about two thirds of the sera tested.

The reason for the lack of neutralization of TSHR autoantibody activity in some sera is unknown and requires further investigation. Possible explanations include 1) insufficient concentration of TSHR antigen relative to the amount of TSHR autoantibody present in serum, 2) varying affinities for antigen of TSHR autoantibodies in different patients, and 3) epitopic differences between TSHR autoantibodies. At present it is not possible to determine the precise epitopes, concentration, and affinity of TSHR autoantibodies in an individual serum or the concentration of autoantigen-competent TSHR in the 17P-9 supernatant fraction. The amount of this material, detected only by precursor labeling, is insufficient to make purification practical. The greater size of the radiolabeled TSHR-ECD in culture medium relative to that in cells suggests that it may be the immunologically active ECD.

Although the 17P-9 CHO cell soluble fraction could neutralize TSHR autoantibodies, it had no effect on TSH binding, even following Mono-Q ion exchange chromatography (data not shown). This relative potency supports previous findings that autoantibodies recognize the TSHR-ECD generated in

insect cells much more effectively than does TSH (7, 15). If TSH does, indeed, interact with the TSHR-ECD (15, 39, 40), the present data suggest that this is a low affinity interaction. What is clear, however, is that TSH does not bind to the high mannose form of the TSHR-ECD. It is intriguing that the closely related ligands, hCG and LH, bind with high affinity to the LH/CG receptor ECD, even with immature, high mannose carbohydrate (41). This difference between the TSH and LH/CG receptors is consistent with other observations of a lesser role for glycosylation in LH/CG (42–44) binding to its receptor than is the case for TSH (45).

A final, but important, point requires emphasizing. The present data indicate that the specificity of the antibody used for detection is critical if the goal is to purify a fully processed, properly glycosylated receptor capable of being recognized by TSHR autoantibodies in patients' sera. The two antisera that we tested were inadequate for recognition of the functional TSHR, perhaps because the initial material used for immunization did not have fully mature carbohydrate moieties. Other available rabbit or murine antibodies should be tested for the specificity of their TSHR recognition. Our findings make even more urgent the as yet unattained quest for cloning high affinity IgG class human autoantibodies to the TSHR.

Acknowledgments

References

1. McLachlan SM, Rapoport B. 1993 Recombinant thyroid autoantigens: the keys to the pathogenesis of autoimmune thyroid disease. J Intern Med. 234:347–359.
2. McLachlan SM, Rapoport B. 1992 The molecular biology of thyroid peroxidase: cloning, expression and role as autoantigen in autoimmune thyroid disease. Endocr Rev. 13:192–206.
3. Rapoport B, McLachlan SM. 1994 Thyroid peroxidase as an autoantigen in autoimmune thyroid disease: update 1994. In: Negro-Vilar A, Braverman LE, Refetoff S, eds. Endocrine review monographs 3. Clinical and molecular aspects of diseases of the thyroid. Bethesda: The Endocrine Society; 96–102.
4. Loosfelt H, Pichon C, Jolivet A, et al. 1992 Two-subunit structure of the human thyrotropin receptor. Proc Natl Acad Sci USA. 895:3765–3769.
5. Rees Smith B, McLachlan SM, Furmaniak J. 1988 Autoantibodies to the thyrotropin receptor. Endocr Rev. 9:106–121.
6. De Forteza R, Smith CU, Amin J, McKenzie JM, Zakarija M. 1994 Visualization of the thyrotropin receptor on the cell surface by potent autoantibodies. J Clin Endocrinol Metab. 78:1271–1273.
7. Harfst E, Johnstone AP, Nussey SS. 1992 Characterization of the extracellular region of the human thyrotropin receptor expressed as a recombinant protein. J Mol Endocrinol. 9:227–236.
8. Takai O, Desai RK, Seetharamaiah GS, et al. 1991 Prokaryotic expression of the thyrotropin receptor and identification of an immunogenic region of the protein using synthetic peptides. Biochem Biophys Res Commun. 179:319–326.
9. Huang GC, Collison KS, McGregor AM, Banga JP. 1992 Expression of a human thyrotropin receptor fragment in *Escherichia coli* and its interaction with the hormone and autoantibodies from patients with Graves' disease. J Mol Endocrinol. 8:137–144.
10. Costagliola S, Alcalde L, Ruf J, Vassart G, Ludgate M. 1994 Overexpression of the extracellular domain of the thyrotrophin receptor in bacteria; production of thyrotrophin-binding inhibiting immunoglobulins. J Mol Endocrinol. 13:11–21.
11. Graves PN, Vlase H, Davies TF. 1995 Folding of the recombinant human thyrotropin (TSH) receptor extracellular domain: identification of folded monomeric and tetrameric complexes that bind TSH receptor autoantibodies. Endocrinology. 136:521–527.
12. Huang GC, Page MJ, Nicholson LB, Collison KS, McGregor AM, Banga JP. 1993 The thyrotropin hormone receptor of Graves' disease: overexpression of the extracellular domain in insect cells using recombinant baculovirus, immunoaffinity purification and analysis of autoantibody binding. J Mol Endocrinol. 10:127–142.

13. Seetharamaiah GS, Desai RK, Dallas JS, Tahara K, Kohn LD, Prabhakar BS. 1993 Induction of TSH binding inhibitory immunoglobulins with the extracellular domain of human thyrotropin receptor produced using baculovirus expression system. Autoimmunity. 14:315–320.

14. Misrahi M, Ghinea N, Sar S, et al. 1994 Processing of the precursors of the human thyroid-stimulating hormone receptor in various eukaryotic cells (human thyrocytes, transfected L cells and baculovirus-infected insect cells). Eur J Biochem. 222:711–719.

15. Chazenbalk GD, Rapoport B. 1995 Expression of the extracellular region of the thyrotropin receptor in a baculovirus vector using a promoter active earlier than the polyhedrin promoter: implications for the expression of functional, highly glycosylated proteins. J Biol Chem. 270:1543–1549.

16. Okamoto Y, Hayashi Y, DeGroot LJ. 1995 Expression of human thyrotropin receptor by recombinant adenovirus. Thyroid. 5:51–53.

17. Nagayama Y, Kaufman KD, Seto P, Rapoport B. 1989 Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor. Biochem Biophys Res Commun. 165:1184–1190.

18. Perret J, Ludgate M, Libert F, et al. 1990 Stable expression of the human TSH receptor in CHO cells and characterization of differentially expressing clones. Biochem Biophys Res Commun. 171:1044–1050.

19. Harfst E, Johnstone AP, Gout I, Taylor AH, Waterfield MD, Nussey SS. 1992 The use of the amplifiable high-expression vector pEE14 to study the interactions of autoantibodies with recombinant human thyrotropin receptor. Mol Cell Endocrinol. 83:117–123.

20. Wadsworth HL, Chazenbalk GD, Nagayama Y, Russo D, Rapoport B. 1990 An insertion in the human thyrotropin receptor critical for high affinity hormone binding. Science. 249:1423–1425.

21. Filetti S, Foti D, Costante G, Rapoport B. 1991 Recombinant human TSH receptor in a radioreceptor assay for the measurement of TSH receptor autoantibodies. J Clin Endocrinol Metab. 72:1096–1101.

22. Kaufman KD, Foti D, Seto P, Rapoport B. 1991 Overexpression of an immunologically-intact, secreted form of human thyroid peroxidase in eukaryotic cells. Mol Cell Endocrinol. 78:107–114.

23. Foti D, Kaufman KD, Chazenbalk G, Rapoport B. 1990 Generation of a biologically-active, secreted form of human thyroid peroxidase by site-directed mutagenesis. Mol Endocrinol. 4:786–791.

24. Chen C, Okayama H. 1987 High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol. 7:2745–2752.

25. Laemmli UK. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680–685.

26. Portolano S, Chazenbalk GD, Seto P, Hutchison JS, Rapoport B, McLachlan SM. 1992 Recognition by recombinant autoimmune thyroid disease-derived Fab fragments of a dominant conformational epitope on human thyroid peroxidase. J Clin Invest. 90:720–726.

27. Nagayama Y, Wadsworth HL, Russo D, Chazenbalk GD, Rapoport B. 1991 Binding domains of stimulatory and inhibitory thyrotropin (TSH) receptor autoantibodies determined with chimeric TSH-lutropin/chorionic gonadotropin receptors. J Clin Invest. 88:336–340.

28. Libert F, Lefort A, Gerard C, et al. 1989 Cloning, sequencing, and expression of the human thyrotropin (TSH) receptor: evidence for binding of autoantibodies. Biochem Biophys Res Commun. 165:1250–1255.

29. Misrahi M, Loosfelt H, Atger M, Sar S, Guiochon-Mantel A, Milgrom E. 1990 Cloning, sequencing, and expression of human TSH receptor. Biochem Biophys Res Commun. 166:394–403.

30. Libert F, Ludgate M, Dinsart C, Vassart G. 1991 Thyroperoxidase, but not the thyrotropin receptor, contains sequential epitopes recognized by autoantibodies in recombinant peptides expressed in the pUEX vector. J Clin Endocrinol Metab. 73:857–860.

31. Nagayama Y, Rapoport B. 1992 The thyrotropin receptor twenty five years after its discovery: new insights following its molecular cloning. Mol Endocrinol. 6:145–156.

32. Braun T, Schofield PR, Sprengel R. 1991 Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. EMBO J. 10:1885–1890.

33. Ji I, Ji TH. 1991 Exons 1–10 of the rat LH receptor encode a high affinity hormone binding site and exon 11 encodes G-protein modulation and a potential second hormone binding site. Endocrinology. 128:2648–2650.

34. Tsai-Morris CH, Buczko E, Wang W, Dufau ML. 1990 Intronic nature of the rat luteinizing hormone receptor gene defines a soluble receptor subspecies with hormone binding activity. J Biol Chem. 265:19385–19388.

35. VuHai-LuuThi MT, Misrahi M, Houllier A, Jolivet A, Milgrom E. 1992 Variant forms of the pig lutropin/choriogonadotropin receptor. Biochemistry. 31:8377–8383.

36. Fiedler KI, Simons K. 1995 The role of N-glycans in the secretory pathway. Cell. 81:309–312.

37. Johnstone AP, Cridland JC, DaCosta CR, Harfst E, Shepherd PS. 1994 Monoclonal antibodies that recognize the native human thyrotropin receptor. Mol Cell Endocrinol. 105:R1–R9.

38. Nagayama Y, Wadsworth HL, Chazenbalk GD, Russo D, Seto P, Rapoport B. 1991 Thyrotropin-luteinizing hormone/chorionic gonadotropin receptor extracellular domain chimeras as probes for TSH receptor function. Proc Natl Acad Sci USA. 88:902–905.

39. Shi Y, Zou M, Parhar RS, Farid NR. 1993 High-affinity binding of thyrotropin to the extracellular domain of its receptor transfected in Chinese hamster ovary cells. Thyroid. 3:129–133.

40. Seetharamaiah GS, Kurosky A, Desai RK, Dallas JS, Prabhakar BS. 1994 A recombinant extracellular domain of the thyrotropin (TSH) receptor binds TSH in the absence of membranes. Endocrinology. 134:549–554.

41. Thomas DM, Segaloff DL. 1994 Hormone-binding properties, and glycosylation pattern of a recombinant form of the extracellular domain of the luteinizing hormone/chorionic gonadotropin receptor expressed in mammalian cells. Endocrinology. 135:1902–1912.

42. Liu X, Davis D, Segaloff DL. 1993 Disruption of potential sites for N-linked glycosylation does not impair hormone binding to the lutropin/choriogonadotropin receptor if Asn-173 is left intact. J Biol Chem. 268:1513–1516.

43. Petäjä-Repo UE, Merz WE, Rajaniemi HJ. 1993 Significance of the carbohydrate moiety of the rat ovarian luteinizing-hormone/chorionic-gonadotropin receptor for ligand-binding specificity and signal transduction. Biochem J. 292:839–844.

44. Tapanainen JS, Bo M, Dunkel L, Billig H, Perlas EA, Boime I, Hsueh AJW. 1993 Deglycosylation of the human luteinizing hormone receptor does not affect ligand binding and signal transduction. Endocr J. 1:219–225.

45. Russo D, Chazenbalk GD, Nagayama Y, Wadsworth HL, Rapoport B. 1991 Site-directed mutagenesis of the human thyrotropin receptor: role of asparagine-linked oligosaccharides in the expression of a functional receptor. Mol Endocrinol. 5:29–33.